Phenotypic and genotypic comparison between pathogenic Group B Streptococcus revealed the possibility of cross transmission between fish and human isolates in Malaysia

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Abstract

Group B *Streptococcus* (GBS) is a leading cause of various infectious diseases among humans and fish. However, the likelihood of GBS interspecies transmission that lead to potential zoonotic problem is less investigated. This study determined the antimicrobial susceptibility, serotype, virulence and pili genes, and sequence type (ST) of GBS isolated from humans and cultured tilapia in Malaysia. We report an unusual detection of the fish-adapted ST283 in human GBS isolates. Both ST283 obtained from humans and fish had several phenotypical and genotypic characteristics in common, including virulence and pilus genes as well as antimicrobial susceptibility, illustrating the value of monitoring GBS within the One Health scope. The study sheds light on the evolution of the GBS and provides new evidence for the potential interspecies transmission by identifying two human GBS isolates belonging to the variant common in fish hosts.

Introduction

Streptococcus agalactiae (also known as Lancefield group B *Streptococcus* (GBS)) is a Gram-positive coccus that is a member of the human commensal gastrointestinal and genitourinary flora in healthy adults[1]. However, GBS is recognized as one of the significant human pathogens that causes a wide range of clinical diseases such as endometritis, meningitis, septicemia, and soft skin tissue infections in pregnant women, neonates, and immunocompromised people in many countries because of its incredible ability to colonize and infect the human host[1,2]. In the last few years, the incidence of invasive GBS infection has increased worldwide, especially among significant underlying medical conditions such as diabetes mellitus, heart disease, malignancies, and older age[3,4].

Moreover, GBS is an important pathogenic bacterium causing infections in cultured fishes worldwide, including economically important cultured fishes such as tilapia (*Oreochromis* sp.), channel catfish (*Ictalurus punctatus*) and rainbow trout (*Oncorhynchus mykiss*)[4]. The disease is commonly affecting various tilapia-producing countries in Southeast Asia, including Malaysia, Indonesia, Vietnam and Thailand, where the mortality can be as high as 70% that lead to significant economic losses and posing a threat to the aquaculture industry's development in this region[5-7].

To elucidate the GBS population structure in relation to disease outbreaks in humans and fish, several phenotypic and genotypic techniques have been developed[1]. GBS virulence is determined by the capsular polysaccharide (*cps*), which promotes adherence to epithelial surfaces of the host and play role in the escape from host defenses that determined by *cps* gene type. GBS can be subtyped into 10 different capsular serotypes, including Ia, Ib, and II to IX, and each serotype possesses a significant virulent capability[8,9]. Serotypes Ia, II, III and V have been characterized as the major strains associated with invasive disease in pregnant women, neonates and non-pregnant adults, while serotypes Ia, Ib and III are commonly associated with fish infection[1,7,10,11]. However, the distribution and predominance of certain serotypes varies over time and differ with a geographical area[8].

Multilocus sequence typing (MLST) is a genotypic method that provides GBS strains' genetic characterization. MLST revealed to GBS consists of 1,349 sequence types (ST) worldwide[12]. Strains belonging to ST1, ST23 and ST19 are the most common in human clinical isolates, while the STs found in GBS isolated from fishes include ST7 and ST283 in Malaysia [6,13].Additionally, GBS virulence gene profiles are determined by genotypic characterization based on virulence regiment, including capsular polysaccharides, surface proteins, toxins and pili that are found in human and fish isolates, which can play a role in immune evasion, adhesion and invade the host cells[1,14]. Previous studies reported that there is a variation in genetic profiles between human and fish GBS[15,16]. Based on the genetic variations, human GBS isolates have gradually become resistant to clindamycin, azithromycin and erythromycin and differ in resistance rate among countries and sources, while the resistance rate to erythromycin, clindamycin, and azithromycin among fish GBS isolates was less than human isolates[10,11,17,18].

GSB has long been associated with transmission from mother to baby (human to human) rather than fish to human (or *vice versa*), although fish may be the reservoir for GBS as well[1,19]. However, in 2015, Singapore experienced its first reported foodborne outbreak of GBS infections[20]. The Singapore GBS outbreak was associated with sequence type (ST) 283 and contaminated raw fish salad consumption. This outbreak was unique because it affected non-pregnant and younger adults with fewer co-morbidities, suggesting more prominent virulence[20,21]. Before 2015, GBS ST283 had been rarely reported in humans but commonly found in diseased

fish[15,22]. Therefore, the fish-borne outbreak has sparked its zoonotic potential. Results from this study are very significant as an early warning to the likelihood of GBS interspecies transmission. Due to the public health and economic significance, this study determined the phenotypic and genotypic variation among GBS isolated from humans and fish in Malaysia for understanding its zoonotic and virulent potential.

Results

Demographic data

A total of 113 invasive human GBS isolates were obtained during the collection period from the six major hospitals in Malaysia; 39 (34.95%) were from Hospital Sultanah Aminah, 22 (19.5%) from Hospital Serdang, 22 (19.5%) from Universiti Kebangsaan Malaysia Medical Center, 15 (13.3%) from Hospital Tuanku Jaafar, 13 (13.3%) from Hospital Melaka and 2 (1.8%) from Hospital Sungai Buloh.

The most common site of isolation was blood (53; 46.9%), followed by internal tissue (35; 31%), pus aspirate (14; 12.4%), peritoneal dialysis fluid (4; 3.5%), bone biopsy (3; 2.6%), cerebrospinal fluid (2; 1.8%), skin biopsy (1; 0.9%) and bone marrow (1; 0.9%). GBS isolates were higher in female (n = 69; 61.1%) than in male patients (n = 44; 38.9%). The ages of the patients ranged from one day to 84 years old. The mean (SD) age was 42.3 (22.6) years old. Most of the patients were non-pregnant adults (87; 77%), followed by neonate (13; 11.5%), pregnant women (10; 8.8%), and infant (3; 2.7%). One or more underlying conditions were present in 74 (62.7%) of the patients. The most common underlying conditions were diabetes mellitus (48.3%), respiratory disease (6.8%), renal disease (5.1%), cardiovascular disease (3.4%), skin and soft tissue disease (3.4%) and neurologic disease (2.5%).

Fish isolates have been previously characterized for phenotypic characteristics and revived for this study for determining the genotypes, which was not done in the previous study[23]. The majority of the sample sources were brain (43%), followed by kidney (35.1%), eye (19.13%) and liver (2.6%) of the fish. The majority of the fishes have internal abnormal necropsy (56.1%). Infected fish showed enlarged gall bladder, peritonitis, liver abnormality, brain lesions, kidney lesions, anaemia, haemorrhagic eye and pale or rotting gill.

Phenotypic characteristics of GBS isolates

Figure 1 shows the phenotypic characteristic of representative GBS isolates on blood agar, CHROMAgar, and GBS brilliance agar, CAMP test, catalase test, grouping agglutination and GBS Latex agglutination test results. All GBS isolated from humans and fish showed grey to white colonies with ß hemolysis on blood agar and pink colonies on CHROMAgar and GBS brilliance agar. All the isolates were positive for the CAMP test, catalase-negative and positive for GBS using the Latex agglutination test. There were no phenotypic differences between the GBS human and fish isolates. The human isolates appeared more typical (strong reaction) of GBS in the Latex agglutination test than the fish isolates.

Antimicrobial susceptibility profiles

Table 1 shows the comparison of antimicrobial susceptibility profiles for 13 antibiotics between human and fish isolates. There were significant differences ($p < 0.05$) between human and fish isolates, where the resistance rate among human isolates for erythromycin (15%), azithromycin (13.3%), clindamycin (8.8%), chloramphenicol (2.7%) and ofloxacin (0.9%) were higher than fish isolates which are susceptible to the antibiotics. However, the tetracycline resistance rate among fish was (98.5%) higher than 75.3% among human isolates. In addition, all the human and fish isolates were susceptible to penicillin, vancomycin, ceftriaxone, cefotaxime, cefepime, and linezolid.

Table 1
The comparison of antimicrobial susceptibility profiles for 13 antibiotics between human and fish isolates

Antibiotics	GBS						p - value
	Human isolates			Fish isolates			
	S	I	R	S	I	R	
P	100.0%	0.0%	0.0%	100.0%	0.0%	0.0%	
AMP	98.2%	1.8%	0.0%	100.0%	0.0%	0.0%	0.2000[a]
DA	91.2%	0.0%	8.8%	100.0%	0.0%	0.0%	0.001*[a]
E	84.1%	0.9%	15.0%	100.0%	0.0%	0.0%	< 0.001*[a]
VA	100.0%	0.0%	0.0%	100.0%	0.0%	0.0%	
TE	18.6%	7.1%	74.3%	4.40%	6.1%	89.5%	0.001*[a]
AZM	85.8%	0.9%	13.3%	100.0%	0.0%	0.0%	< 0.001*[a]
CRO	100.0%	0.0%	0.0%	100.0%	0.0%	0.0%	
CTX	100.0%	0.0%	0.0%	100.0%	0.0%	0.0%	
FEP	100.0%	0.0%	0.0%	100.0%	0.0%	0.0%	
OFX	98.25	0.9%	0.9%	100.0%	0.0%	0.0%	0.180[a]
C	96.5%	0.9%	2.7%	100.0%	0.0%	0.0%	0.050*[a]
LZD	100.0%	0.0%	0.0%	100.0%	0.0%	0.0%	

*$p > 0.05$ = statistically significant, ([a]p) = Fisher's Exact Test; P: penicillin G, AMP: ampicillin, E: erythromycin, DA: clindamycin, AZM: azithromycin, C: chloramphenicol, OFX: ofloxacin, FEP: cefepime, CTX: cefotaxime, CRO: ceftriaxone, TE: tetracycline, LZD: linezolid and VA: vancomycin.

Distribution of serotypes

The results of serotype distribution showed variation between human and fish GBS isolates (Table 2). Among the human isolates, the most common serotype was serotype V ($n = 26$; 23%), followed by Ia ($n = 22$; 19.5%), II ($n = 21$; 18.6%), VI ($n = 18$; 15.9%), III ($n = 12$; 10.6%), VII ($n = 5$; 4.4%), Ib and IV, ($n = 4$; 3.5% each) and IX ($n = 1$; 0.9%). While all the fish isolates have serotype III ($n = 114$; 100%). This variation was statistically significant ($p < 0.05$) for serotype III.

Table 2
Serotype distribution of GBS isolates among human and fish

Serotypes	GBS	
	Human isolates	Fish isolates
Ia	22 (19.5%)	0 (0%)
Ib	4 (3.5%)	0 (0%)
II	21(18.6%)	0 (0%)
III	**12 (10.6%)**	**114 (100%)***
IV	4 (3.5%)	0 (0%)
V	26 (23.0%)	0 (0%)
VI	18 (15.9%)	0 (0%)
VII	5 (4.4%)	0 (0%)
IX	1 (0.9%)	0 (0%)

*$p > 0.05$ = statistically significant, *as only one serotype identified in fish isolates, statistic was done only on this serotype III with $p = < 0.001$ (bold).

Virulence and pilus genes profiles

The comparison of virulence gene profile and detection of each gene between the human and fish isolates is shown in Fig. 2. The prevalent gene profiles were *cfb* (100% vs. 100%), *cylE* (100% vs. 100%), *hylB* (96.5% vs. 99.1%), *fbsA* (100% vs. 100%), *spb1* (99.1% vs. 100%) and *bca* (83.2% vs. 91.2%) in the human and fish isolates.

We observed a significantly decreased rate of *rib* (5.1% vs. 100%; $p < 0.001$), *scpB* (5.3% vs. 100%; $p < 0.001$), PI-2b (8.8% vs. 30.1%; $p < 0.001$), *lmb* (21.1% vs. 100%; $p < 0.001$) and PI-2a (0.0% vs. 70.8%; $p < 0.001$) in the fish isolates. We also found a significantly decreased prevalence of *bac* (12.4% vs. 99.1%; $p < 0.001$), *fbsB* (95.6% vs. 100%; $p < 0.03$) and PI-1 (86.7% vs. 100%; $p < 0.001$) in the human isolates compared to that in fish isolates.

Multilocus sequence typing analysis

The sequences of the seven housekeeping genes were amplified in 46 human GBS isolates and 15 fish GBS isolates. The MLST analysis determined the presence of allelic profile and sequence type (ST) of 19 different STs and eight different clonal complexes (CC) (Table 3). Among the human GBS isolates, the predominant ST were ST1 ($n = 15$, 32.6%), followed by ST17 ($n = 6$, 13%), ST3, ST12 and ST26 ($n = 3$, 6.5% for each), ST24 and ST283 ($n = 2$, 4.3% for each), ST19, ST23, ST28, ST130, ST196, ST335, ST459, ST485 and ST861 ($n = 1$, 2.2% for each), and lastly three novel sequence types ST1668, ST1669 and ST1670 ($n = 1$, 2.2% for each).

Table 3
Genotypic characteristics of GBS isolates among human and fish concerning serotypes and antibiotic susceptibilities

ST	CC	GBS		Serotype									Number of isolates resistant to antibiotics						MDR (n)
		Human	Fish	Ia	Ib	II	III	IV	V	VI	VII	IX	DA	E	TE	AZM	OFX	C	
1	CC1	15	0	1	1	1	0	0	2	6	4	0	2	2	9	2	0	0	Yes (2)
196		1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	No
459		1	0	0	0	0	0	1	0	0	0	0	1	1	0	1	0	0	Yes (1)
1668		1	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	No
1669		1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	No
3	CC3	3	0	0	0	1	2	0	0	0	0	0	1	1	3	1	0	0	Yes (1)
12	CC12	3	0	0	3	0	0	0	0	0	0	0	3	3	3	3	0	2	Yes (3)
1670		1	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	No
485		1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	No
130		1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	No
17	CC17	6	0	0	0	0	6	0	0	0	0	0	2	3	6	2	0	0	Yes (2)
19	CC19	1	0	1	0	0	0	0	0	0	0	0	0	1	1	1	1	0	Yes (1)
335		1	0	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	Yes (1)
861		1	0	0	0	0	1	0	0	0	0	0	1	1	1	1	0	0	Yes (1)
28		1	0	0	0	1	0	0	0	0	0	0	0	1	1	1	0	1	Yes (1)
23	CC23	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	No
24		2	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	No
26	CC26	3	0	0	0	0	0	0	3	0	0	0	0	0	3	0	0	0	No
283	CC283	2	15	0	0	0	17	0	0	0	0	0	0	0	14	0	0	0	No

ST = sequence type; CC = clonal complex; E = erythromycin, DA = clindamycin, AZM = azithromycin, C = chloramphenicol, OFX = ofloxacin, TE = tetracycline; MDR = multidrug resistance.

The novel ST1668, ST1669 and ST1670 originated from the tissues of a 70-year-old female, a 76-year-old female, and a 47-year-old male, respectively. On the other hand, all the randomly selected 15 fish isolates were ST283. MLST has demonstrated that two human GBS isolates have ST283 similar to the fish GBS isolates. All the human and fish GBS ST283 were serotype III, susceptible to all antibiotics except the tetracycline. The human GBS ST283 were isolated from blood and pus aspirate, Chinese ethnicity and patients without significant comorbidities.

Serotype III was found in ST283 (*n* = 17), ST17 (*n* = 6), ST3 (*n* = 2), and ST335 with ST861 *(n* = 1 for each). Serotype V was found in ST26 (*n* = 3) and ST1 (*n* = 2). Serotype VI was found in ST1 (*n* = 6) and ST24 with ST1668 (*n* = 1 for each). Serotype VII was found in ST1 (*n* = 4), while serotype Ia was found in ST1, ST19, ST23, ST24, St485 and ST1670 (*n* = 1 for each). Serotype Ib was found in ST12 (*n* = 3) and ST1 (*n* = 1). Serotype II was found in ST1, ST3, ST28 and ST1669 (*n* = 1 for each). Serotype IV was found in ST196 and ST459 (*n* = 1 for each). Serotype IX was found in ST130 (*n* = 1).

In PHYLOViZ analysis, STs which share six or more identical alleles are categorized into one group which indicates a clonal complex (CC). The ST with largest single locus variant (SLV) in a group is assigned as a primary founder of the group. The CCs are named after the primary founder. In a case where two STs have a same SLV number, the one with higher double locus variant (DLV) number is chosen as a primary founder. The PHYLOViZ analysis revealed eight different major CCs from 19 different STs. The most predominant CC was CC1 (n = 19) followed by CC283 (n = 17), CC12 and CC17 (n = 6 for each), CC3, CC23 and CC26 (n = 3 for each). ST1, ST196, ST459, ST1668 and ST1669 were grouped into CC19. ST3 was assigned to CC3. ST12, ST1670, ST485 and ST130 were categorized under CC12. ST17 was assigned to CC17. ST19, ST335, ST861 and ST28 were grouped into CC19. ST23 and ST24 were categorized under CC23, while ST26 was defined as CC26 and ST283 as CC283.

Phylogenetic analysis

All nucleotide sequences of the seven housekeeping genes for the respective isolates were aligned in the specified order and subjected to phylogenetic analysis among 61 GBS isolates (46 human isolates and 15 fish isolates) (labelled with the abbreviation of hospital name for human isolates and letter F for fish isolates followed by sample numbers) and 16 reference sequences from the MLST database (labelled with REF followed by identity number). The tree was basically rooted in clade IV) and branched out into three classes: I, II, and III, with 100% bootstrap confidence interval values at all branching points (Fig. 3).

Among the four clades, two large groups of 27 isolates (clade I) and 24 isolates (clade II) from this investigation, whereas the other two were small clades with four isolates (clade III) and nine isolates (clade IV). Among the STs, the clades showed clear segregation, where clade I represented ST283, ST12, ST130, ST1670 (novel), ST485, and ST3; clade II for ST28, ST861, ST335, ST19, ST1668 (novel), ST196, ST459, ST1 and ST1669 (novel); clade III for ST26; clade IV for ST24, ST23 and ST17. All reference sequences of the different STs were clustered according to their respective comparable ST of the isolates in this investigation, except the new STs.

Discussion

Although many studies reported the importance of GBS in human and veterinary medicine transmission between human and animal or *vice versa* is poorly understood[24]. Furthermore, molecular epidemiological studies that compare the phenotype and genotype properties of GBS involving different origins, particularly human and fish origin, are scarce in South East Asia.

The multidrug-resistant isolates have been increasing due to the intensive culture of fish and antimicrobial misuse to control bacterial infection in South East Asia. According to the Centers for Disease Control and Prevention (CDC) in the US, GBS isolates are completely susceptible to beta-lactam antibiotics include penicillin derivatives and cephalosporins. At the same time, CLSI reports that non-susceptible GBS isolates to beta-lactams are sporadic[25]. Despite this, penicillin resistance in GBS has been reported from Asia, including in human isolates in Japan and from tilapia isolates in China[26,27]. GBS antimicrobial resistance profiles differed among humans and fish origin.

In this study, we reported resistance to erythromycin, azithromycin, clindamycin, chloramphenicol and ofloxacin among human GBS isolates, while all fish GBS isolates were susceptible to these antibiotics. This contrasts with results reported from China, where erythromycin resistance was higher among GBS isolated from cultured fish[28,29]. In contrast, the fish isolates were higher in resistance to tetracycline in this study which may be because tetracycline is widely used to treat several bacterial infections in freshwater fish[30]. These results were similar to previous studies in Southeast Asia among human and fish origin[31,32].

In addition, the antimicrobials penicillin, amoxicillin, vancomycin, ceftriaxone, cefotaxime, cefepime, and linezolid showed largely efficacy against humans and fish GBS isolates, which were reported in previous studies as well[11,18,33–35]. Penicillin is the first-line antibiotic for treating and prophylaxis GBS infections among humans; however, erythromycin and clindamycin are the alternative antibiotics for allergic patients allergic to penicillin[35]. In our study, the resistance rates to erythromycin and clindamycin among human isolates were lower than those reported in other studies in major hospitals in Malaysia[10,11]. An increase of resistance to erythromycin, clindamycin and azithromycin are commonly used in humans may be needed to concern. GBS human isolates had a common multidrug resistance pattern of resistance to these antibiotics, which was also identified in other studies[10,11,35].

In this study, *cps* gene serotyping revealed that there was a difference in serotype distribution among GBS human and fish isolates in this study. There was a different variety of serotypes among the human isolates. The most common serotype was serotype V, followed by Ia, II, VI and III. Furthermore, serotype VIII did not occur among the human isolates, while serotype Ix was the lowest. Previous studies showed serotypes V and Ia as the common serotypes among GBS human invasive isolates from sterile isolation sites [9,11,36]. However, serotype III was the most common (87.15%), and it was detected mainly in septicemia cases among invasive GBS in Thailand. In another study in Malaysia, the most prevalent serotype from sterile and non-sterile isolation sites VI, followed by VII, III, Ia and V[10]. Generally, serotype Ia and V are commonly present in non-pregnant adults[37]. In addition, serotypes III and V are usually associated with invasive GBS infections[38]. On the other hand, only serotype III was identified from all 114 fish GBS isolates. Other studies reported serotype III was dominant in tilapia in Malaysia, Thailand, Vietnam and Brazil[7,39–42]. However, a study in Brazil reported that was serotype Ib was the dominant[34], while another study in China found serotype Ia to be the dominant[44]. Moreover, a similar was found between serotype III isolated from red tilapia with the GBS found in the Singaporean outbreak, possibly due to cross-host pathogenesis of the strains. Globally, there are marked regional differences in the distribution of GBS serotypes.

The virulence and pili genes promote GBS to attack, destroy and invade the host cells and evade the immune system. The present study showed that *cfp, cylE, hylB, fbsA, fbsB* and *spb1* genes were present in nearly 100% of the human and fish isolates. We observed a difference between GBS human and fish isolates in the possession of virulence genes *lmb*, *scpB*, *rib*, *bac*, PI-1, PI-2a and PI-2b. The *scpB*, *lmb* and *rib* genes are usually found in human GBS isolates and absent in fish and bovine isolates[45]. PI-2a was completely missing from the fish isolates, similarly to previous[46]. This difference is probably due to deletion during putative adaptation of GBS to the fish host[45,47]. Thus, it might be possible to characterize the strains of different host origins using the virulence genes profiles. According to the virulence genes profiling of GBS, human isolates are believed to possess a higher capability to adhere and invade the host cells compared to fish isolates. However, the immune evasion functions of fish isolates could be higher than human isolates.

This study revealed 19 different STs among human and fish isolates, including three newly assigned STs. ST1 was the most prevalent among the human isolates and belonged to CC1, while only ST283 was found in all fish isolates. This is in line with a study in Malaysia that reported ST1 was the most common ST associated with invasive human isolates[13]. Another study in Taiwan also reported that ST1 (26.0%) was the most prevalent STs as a leading cause of invasive disease that affects non-pregnant adults[43]. ST283 were the common STs related to epidemiological studies and disease outbreaks in fish[42,44,48]. In Malaysia, a study of MLST assays showed that eight and 50 isolates of GBS were represented by ST7 and ST283 only, respectively[7]. MLST data of GBS from fish in Malaysia is still lacking, as well as for human isolates. According to a study conducted in China, GBS ST7 has been identified as a highly virulent strain that causes meningoencephalitis in tilapia[49]. In addition, Thailand has reported the case of GBS III ST283 infection in tilapia, which causes significant economic losses to their aquaculture industry[6]. In the current study, GBS III ST283 was found in two cases among the human isolates, which were associated with Chinese ethnicity, non-pregnant patients and susceptibility to antibiotics. Similar studies reported that GBS ST283 was more associated with eating raw fish, Chinese ethnicity and non-pregnant patients[20,21]. To our knowledge, we report GBS III ST283 in humans for the first time in Malaysia. Meanwhile, GBS ST283 has also been isolated from human clinical samples in Thailand has demonstrated which were closely related to the Singaporean outbreak strain in 2015, and it has been demonstrated that ST283 is a zoonotic GBS clone that causes severe invasive disease in humans[21]. CC283 was associated with the type B gbs2018 gene emergence as a human pathogen[50]. This highlights the threat of continued evolution and expansion of the host range of GBS. Furthermore, CC1 was identified as a common cause of GBS septicemia induced by consuming contaminated seafood in France[51]. Based on the examples presented here, it is evident that there are no true species barriers, at least at the ST level. The risk of transmission of GBS between fish and people can be reduced by improving hygiene and cooking food properly.

The phylogenetic tree indicated a genetic linkage between two human and fish isolates sharing a similar lineage of CC283 in clade I. All isolates in CC283 showed non-multidrug resistance pattern and serotype III as well, suggesting that the strains isolated from the human was likely to originate from fish. In bacteria, diversity is associated with mutations, transformation, transduction, or conjugation involving horizontal DNA transfer. GBS genetic diversity was primarily due to recombination, which can lead to altered serotypes, virulence, and host range[52,53]. Our results also placed CC12 and CC3 near CC283 on the phylogenic tree. The most dominant clade I consisted of isolates that exhibited ST283, ST12, ST130, ST1670, ST485, and ST3 with different serotypes;

serotype III was the most prevalent in clade I. Clade II consisting of human GBS alone isolates, was the second major clade representing ST28, ST861, ST335, ST19, ST1668, ST196, ST459, ST1 and ST1669. There was a difference in serotype distribution and MDR phenotype in this clade. Meanwhile, clade III consisted of three ST26 isolates with similar serotype V, non-MDR, and they were both from blood, which reflected their close association. At the same time, clade IV represented ST24, ST23 and ST17. The most common serotype in this clade was III, followed by Ia. All the isolates in this clade were non-MDR. The new ST1670 belonged to the CC12 lineage, while ST1668 and ST1669 belonged to CC1 in the phylogenetic tree, having serotype Ia, VI and II, respectively, and non-MDR. CC17 could emerge differently from others as it was located more basally and distinct in the tree structure. In this study, phylogenetic tree is consistent with previous study[54], in which GBS isolated from different hosts exhibited high genetic diversity, with few relationships between genotypes. All in all, of the human isolates, there are only two GBS ST 283. Therefore, these strains should be monitored sequentially throughout Malaysia, and the additional fish-origin isolates need to be characterized. Further analysis utilizing whole-genome sequencing is highly warranted to elucidate this matter and provide a better understanding of the genetic organization and evolution of the human and fish isolates.

Conclusion

This study indicates similar phenotypic characteristics and different features in virulence gene profile, AMR, and sequence type between GBS from human and fish origins. Fish GBS isolates have less genetic diversity and higher antimicrobial susceptibility compared to human isolates. However, the characterization of GBS ST283 isolates from fish using serotyping, AMR, virulence gene profile, and sequence type determination suggested a relationship with human origin. GBS ST283 has been detected in both humans and fish, suggesting that this group of genetic lineage has been transmitted in both hosts, but the number of cases involving humans is still small. The evidence shows that GBS interspecies transmission, at least under certain conditions, seems possible. GBS may evolve to switch between human and animal hosts, acquiring accessory genome content from different niches. The analysis of GBS isolates with whole-genome sequencing may help to answer these and other questions regarding the possibility of interspecies transmission and GBS evolution.

Materials And Methods

Bacterial isolates

A total of 113 invasive GBS isolates were collected from six Malaysian hospitals, including Hospital Tuanku Jaafar (HTJ), Seremban, Negeri Sembilan; Serdang Hospital (HS), Selangor; Hospital Sultanah Aminah (HSA), Johor; Universiti Kebangsaan Malaysia Medical Center (UKM), Kuala Lumpur; Hospital Melaka (HM), Melaka; and Hospital Sungai Buloh (HSB), Selangor (collection period from November 2019 to December 2020) (Supplementary Table S1).

In addition, we used a random collection of 114 invasive GBS isolated from cage cultured red hybrid tilapia (*Oreochromis niloticus* × *Oreochromi mossambicus*) that showed either softening of the brain with an accumulation of fluid, or haemorrhages affecting internal organs including spleen, liver and kidney. The isolates were obtained from various important aquaculture sites and geographical origins in Malaysia, including Kedah, Selangor and Terengganu, from our previous studies[23,33,55] (Supplementary Table S2).

The criteria for invasiveness are based on standard interpretation related to human and fish diseases. GBS was re-identified by phenotypic test includes colony morphology, β haemolysis on Columbia Agar with 5% sheep blood (Isolab Sdn. Bhd., Kuala Lumpur, Malaysia), pink colonies on CHROMAgar (Isolab Sdn. Bhd.), and GBS brilliance agar (Isolab Sdn. Bhd.). GBS was confirmed by Gram staining, catalase test, CAMP test and latex agglutination test (Oxoid, Basingstoke, UK). *Streptococcus agalactiae* ATCC 13813 and *Streptococcus agalactiae* ATCC 12386 were used as a positive control in all analyses[10].

Antimicrobial susceptibility test

Disc diffusion (Kirby-Bauer) method was used to determine the antimicrobial susceptibility of the GBS isolates to penicillin, ampicillin, clindamycin, erythromycin, vancomycin, tetracycline, azithromycin, ceftriaxone, cefotaxime, cefepime, ofloxacin, chloramphenicol, linezolid (Oxoid, Basingstoke, UK). We selected antibiotics based on their relevance as the first and alternative treatment for GBS infections in Malaysian hospitals and their widely use in aquaculture[28,56]. Meanwhile, the E test method was

used to determine the minimal inhibitory concentrations (MIC) of resistant strains. The bacterial suspension for respective isolates was prepared in 0.9% saline, equivalent to a 0.5 McFarland standard and inoculated on Mueller Hinton Agar (MHA) with 5% sheep blood (Isolab Sdn. Bhd.). Plates incubated at 37ºC in 5% CO_2 for 18 to 20 h[10,11]. The diameter of inhibition zones was measured, and MIC was read based on the recently stated CLSI guideline[25]. *Streptococcus pneumonia* ATCC 49619 was used as quality control recommended by CLSI guideline[25].Multidrug-resistant (MDR) isolates were characterized as those that were resistant to three or more antimicrobial agents[57].

Genomic DNA extraction

Following the manufacturer's instructions, GBS genomic DNA was extracted using the GeneAll® ExgeneTM Mini genomic extraction kit (GeneAll, Seoul, South Korea). NanoPhotometer® was used to determine DNA quantity and quality (Implen, Munich, Germany).

Virulence and pilus genes profiling

The GBS virulence (*cfb, cylE, lmb, scpB, hylB, rib, fbsA, fbsB, spb1, bca* and *bac*) and pili genes (PI-1, PI-2a and PI-2b) were amplified using PCR that performed on a BioRad Thermal Cycler (BioRad, Massachusetts, USA) using the primers and running conditions published previously[34,58–63] (Supplementary Table S3). A total volume of 25 µL reaction mixtures were prepared, containing 12.5 µL master mix Mix (Bioline, London, United Kingdom), 1.0 µL of forward and reverse primer, 3.0 µL of DNA template and 7.5 µL of nuclease-free water. The primers were synthesized by Apical Scientific Laboratory, Kuala Lumpur, Malaysia.

The amplified PCR products were detected by agarose gel electrophoresis on 1.7% agarose gel with Nucleic Acid Stain (Yeastern Bio, Taipei, Taiwan) for 60 min at 80 V. The gel images were visualized using a UV transilluminator (BioRad). The base-pair size of the amplified DNA was confirmed by comparing it with the DNA ladder (Genedirex, Taipei, Taiwan) and the positive control. The valid result of the PCR is confirmed only when the designated band was seen in positive control and absence in the negative control[59]. DNA of *Streptococcus agalactiae* ATCC 13813 was used as a positive control, and a mixture with nucleus water was used as a negative control.

Capsular polysaccharides types

GBS capsular serotypes were deduced by the multiplex PCR method. Ten serotypes (Ia, Ib and II - IX) were determined using published primers according to the study by Imperi et al.[8] (Supplementary Table S4). Capsular polysaccharide synthesis gene (*cpsL*) was used as a positive control was included in each set of primer pairs. For negative control, nuclease water was added to the PCR mixture instead of the DNA template. The primer pairs were arranged in the respective multiplex sets according to their representative serotypes The more common serotypes were targeted first, followed by the fewer ones. The primers were synthesized by Apical Scientific Laboratory, Kuala Lumpur, Malaysia. Multiplex PCR reactions were done in a total volume of 25 µL, consisting of 12.5 µL of PCR master mix MyTaq™ HS Red Mix (Bioline), 3.0 µL of DNA template, 1.0 µL of serotypes specific primer pairs, 4.5 µL of nuclease free water, and 0.5 µL of *cpsL* specific primer pair[8].

Multilocus sequence typing

MLST was conducted on 46 human GBS isolates that is resistant to multiple antibiotics and serotype III and a random selection of 15 GBS isolates of tilapia. The internal fragments of seven housekeeping genes (*ashP, pheS, atr, glnA, sdhA, glcK* and *tkt*) of GBS were amplified by PCR, as previously described[12] (Supplementary Table S5). The PCR product was sent for sequencing at BioBasic, Kuala Lumpur, Malaysia. The sequences of the seven housekeeping genes were trimmed using MEGA 7 software according to known alleles base pair as described in the MLST database website (http://pubmlst.org/) in order to determine the allelic number and sequence type (ST). We have submitted the new alleles and STs to the MLST curator for verification. A clonal complex of obtained ST's was analyzed using the PHYLOViZ software.

MEGA7 software was used to construct MLST phylogenetic tree based on GBS seven housekeeping genes to investigate genetic variation between human and fish GBS isolates using the maximum-likelihood method based on the Tamura-Nei model, and the tree's reliability was determined using bootstrap analysis with 1000 replicates. The MLST database website was used to collect reference sequences ID 3 (United Kingdom), 14 (United Kingdom), 190 (Japan), 194 (Japan), 651 (Japan), 678 (Japan), 1107 (Malawi), 1474 (Singapore), 1771 (Singapore), 4784 (Ireland), 4869 (Ireland), 5708 (Australia), 5896 (Australia), 6121(Australia), 15228 (Singapore), and 15482 (Canada) for respective STs, which were used as controls in the analysis.

Statistical analysis

Chi-square was used to determine the phenotypic and genotypic differences (serotype, virulence gene, pili gene and sequence type) between human and fish GBS isolates. *P*-value at < 0.05 indicated statistical significance (SPSS version 26 for Microsoft Windows, Chicago, USA).

Declarations

Ethics statement

Ethical approval to conduct this study was obtained from the Medical Research and Ethics Committee of the Ministry of Health Malaysia (Approval number: NMRR 19-876-46665). The study used de-identified *streptococcus agalactiae* isolates collected from the microbiology laboratories of the hospitals. Informed consent was not required for this study as it did not meet the definition of research involving human subjects; only data on the isolation site, age, gender and ethnicity of patients associated with the isolates were gathered and could not be tracked back to the individuals sampled. Additionally, all methods were carried out in accordance with the relevant guidelines and regulations.

Data Availability

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

A.R.M and M.N.M.D designed and performed the experiments; W.A and N.A.L.A.A assisted with the experiments; A.R.M, N.D.D and N.H.Z.B analyzed the data; A.R.M and M.N.A.A wrote the manuscript; N.A.A, M.M.A, L.A.M.N, Z.I, N.H.A, C.H.S and M.N.A.A collected the isolates and M.N.M.D and S.A-N supervised the project. All authors read and approved the final manuscript.

Competing interest

The authors declare no competing interests.

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Figures

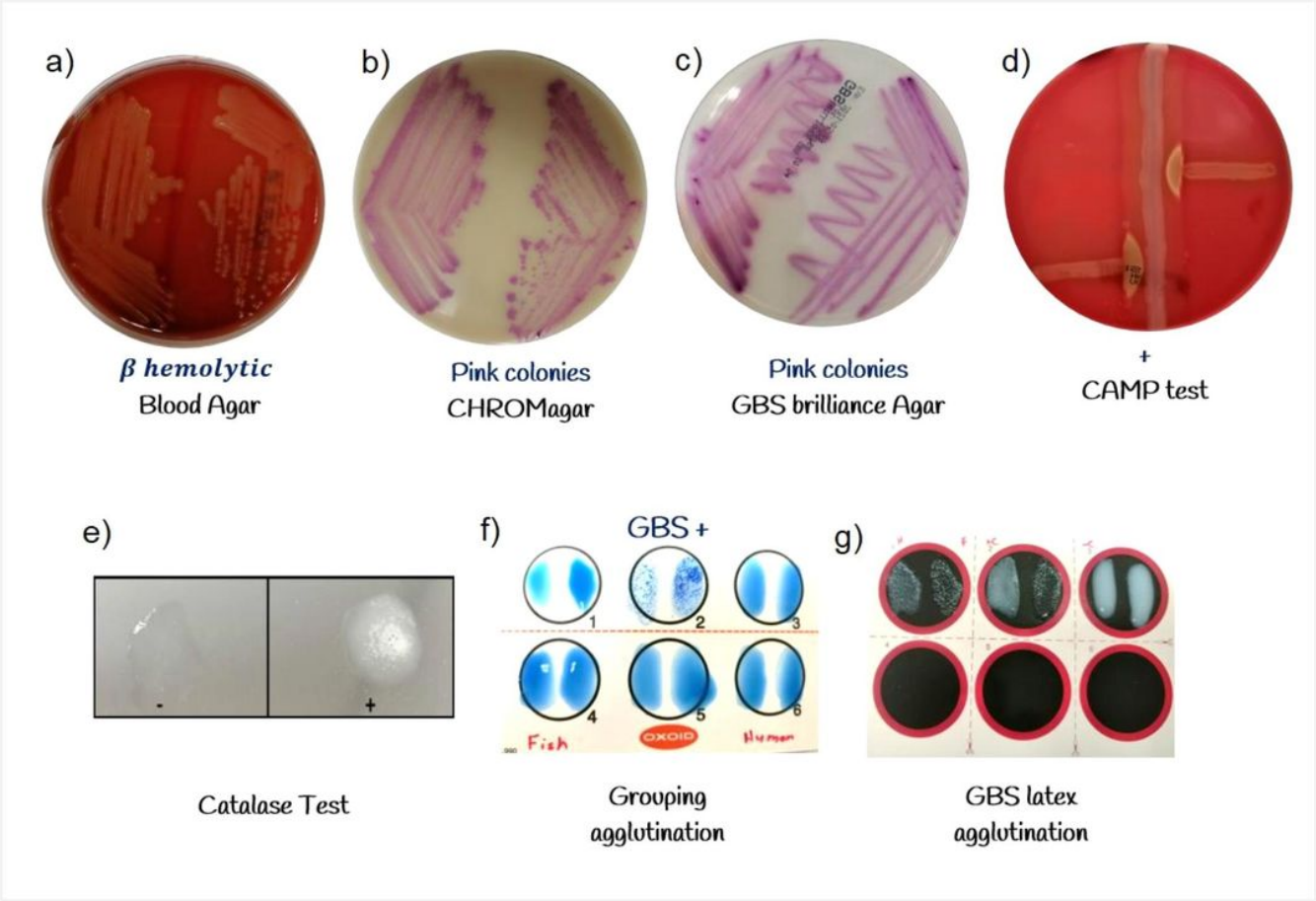


Figure 1

The phenotypic characteristics of GBS isolates. a) appearance of group B *Streptococcus* (*Streptococcus agalactiae*) produces a zone of β hemolytic around the colonies on the blood agar, following 24 hours of incubation under anaerobic conditions; b) pink colonies on CHROMAgar; c) pink colonies on GBS brilliance agar; d) positive CAMP results which indicated by an arrowhead GBS β-hemolytic zone perpendicular to *Staphylococcus aureus*; e) negative catalase result for GBS isolates; f) agglutination in No. 2 identified positive result of GBS and it showed blue Latex particles using a Latex agglutination test; g) positive result of GBS and it showed white Latex particles using a Latex agglutination test.

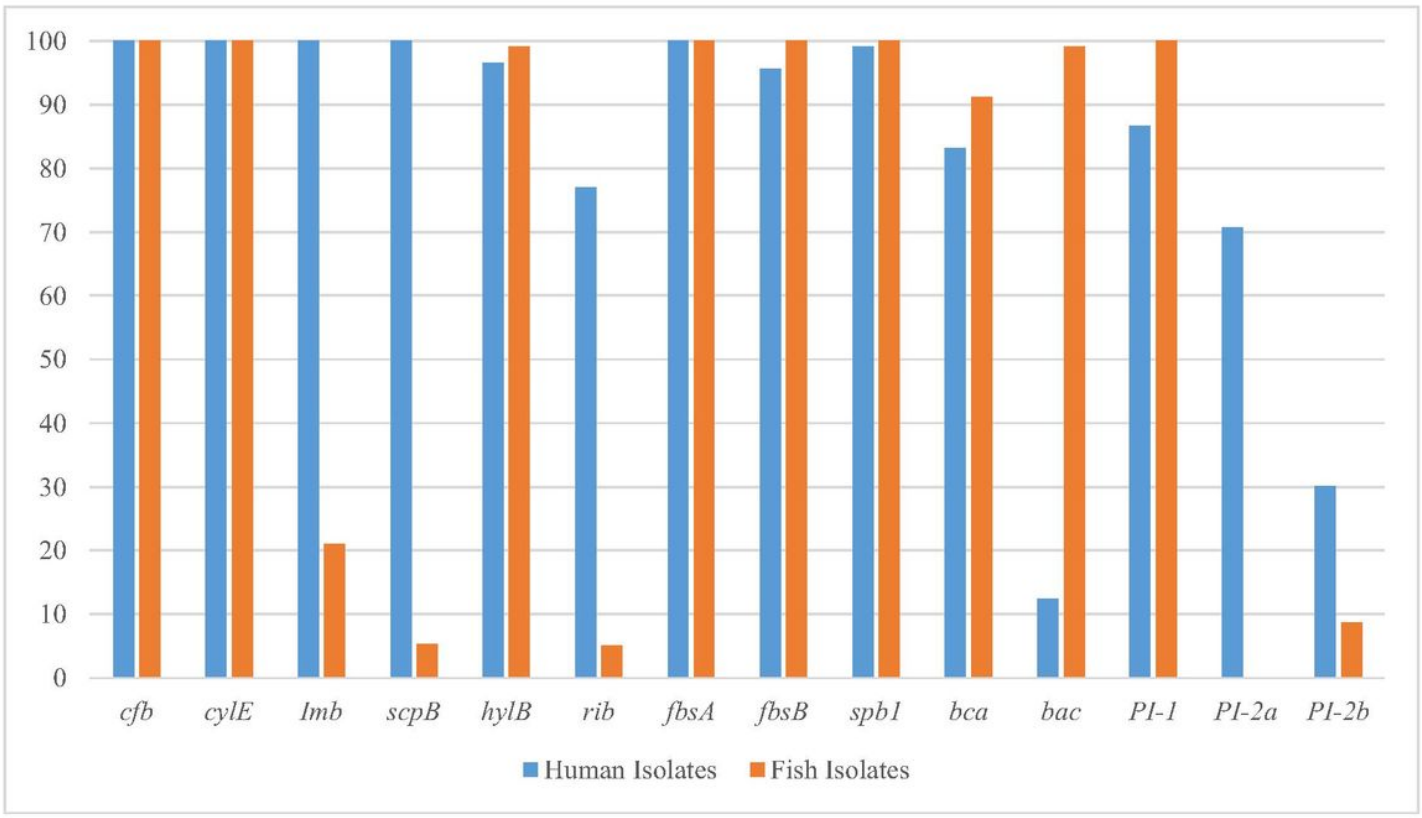
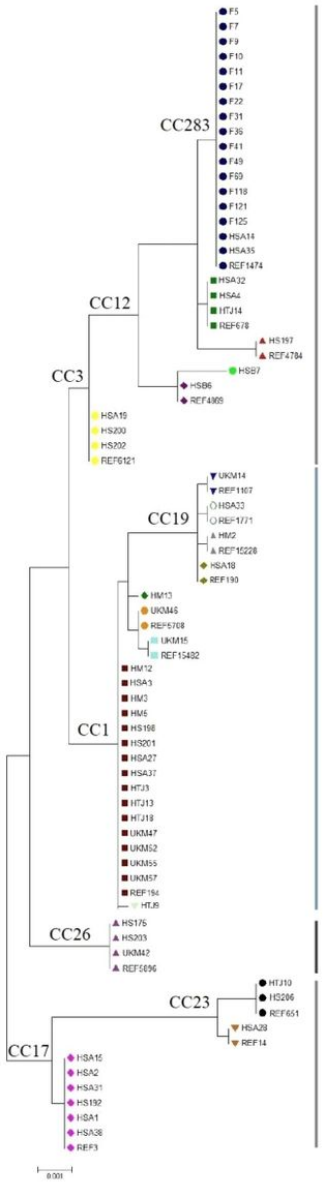


Figure 2

The comparison of virulence and pili genes profiling between GBS human and fish isolates.



Source	ST	Serotype	MDR
Fish (Kidney)	ST283	III	No
Fish (Brain)	ST283	III	No
Fish (Kidney)	ST283	III	No
Fish (Brain)	ST283	III	No
Fish (Kidney)	ST283	III	No
Fish (Brain)	ST283	III	No
Fish (Kidney)	ST283	III	No
Fish (Kidney)	ST283	III	No
Fish (Brain)	ST283	III	No
Fish (Brain)	ST283	III	No
Fish (Brain)	ST283	III	No
Fish (Brain)	ST283	III	No
Fish (Brain)	ST283	III	No
Fish (Kidney)	ST283	III	No
Fish (Liver)	ST283	III	No
Pus aspirate	ST283	III	No
Blood	ST283	III	No
Blood	ST283	III	Not stated
Cerebrospinal fluid	ST12	Ib	Yes
Blood	ST12	Ib	Yes
Internal tissue	ST12	Ib	Yes
Blood	ST12	Ib	Not stated
Blood	ST130	IX	No
Blood	ST130	IX	Not stated
Internal tissue	ST1670	Ia	No
Internal tissue	ST485	Ia	No
Blood	ST485	Ia	Not stated
Blood	ST3	III	No
Internal tissue	ST3	II	Yes
Blood	ST3	III	No
Blood	ST3	II	Not stated
Blood	ST28	II	Yes
Blood	ST28	III	Not stated
Blood	ST861	III	Yes
Blood	ST861	III	Not stated
Blood	ST335	III	Yes
Blood	ST335	III	Not stated
Blood	ST19	Ia	Yes
Cerebrospinal fluid	ST19	III	Not stated
Internal tissue	ST1668	VI	No
Blood	ST196	IV	No
Blood	ST196	Ib	Not stated
Blood	ST459	IV	Yes
Blood	ST459	IV	Not stated
Internal tissue	ST1	V	No
Blood	ST1	VII	No
Blood	ST1	VI	No
Blood	ST1	VI	No
Peritoneal dialysis fluid	ST1	VI	No
Bone biopsy	ST1	VII	No
Blood	ST1	VI	No
Internal tissue	ST1	V	Yes
Blood	ST1	VII	No
Blood	ST1	II	No
Blood	ST1	Ia	No
Blood	ST1	Ib	No
Blood	ST1	VI	No
Internal tissue	ST1	VII	No
Internal tissue	ST1	VI	No
Blood	ST1	V	Not stated
Skin biopsy	ST1669	II	No
Blood	ST26	V	No
Blood	ST26	V	No
Blood	ST26	V	No
Blood	ST26	IV	Not stated
Pus aspirate	ST24	VI	No
Blood	ST24	Ia	No
Blood	ST24	Ia	Not stated
Pus aspirate	ST23	Ia	No
Blood	ST23	Ia	Not stated
Blood	ST17	III	No
Blood	ST17	III	No
Blood	ST17	III	No
Blood	ST17	III	No
Cerebrospinal fluid	ST17	III	No
Blood	ST17	III	No
Blood	ST17	III	Not stated

Figure 3

Phylogenetic analysis of the GBS isolated from human and fish.

Supplementary Files

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- SupplementaryData.docx