Prevelance and Susceptibility Analysis of Gram negative Pathogens

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Abstract: *Antimicrobial resistance is a growing threat worldwide. Predominant mechanism for resistance to the β -lactam antibiotics in Gram negative bacilli is the production of β -lactamases. The present work was aimed to evaluate the antibiotic susceptibility pattern of 368 isolates, isolated from more than 563 clinical samples towards Piperacillin/tazobactam, Imipenem/cilastatin, Amoxacillin/Clavulanic acid and compared its efficacy with a new antibiotic adjuvant entity Elores (ceftriaxone+sulbactam with adjuvant ethylenediaminetetraacetic acid/EDTA). Among the samples which showed the presence of pathogens, around 47.3 % samples were of urine followed by blood, pus and sputum samples which contributed to 36.0 %, 8.7% and 5.2 % respectively. Among the isolates, E. coli (61.4 %) was found to be the most dominant pathogen. Klebsiella species (22.0 %), and P. aeruginosa (14.4 %), also contributed significantly to the isolated pool of pathogens followed by A. baumannii (1.1 %), and P. mirabilis (1.1 %). Higher susceptibility rates were achieved by Elores in comparison with Piperacillin/tazobactam and Amoxacillin/clavulanic acid. Imipenem/cilastatin resistance was high in A. baumannii (75 %) whereas Proteus spp. showed (100 %) susceptibility. Predominant pathogens when compared to Elores to which low resistance ranged from (12.0 %) (least in Proteus spp.) to (22.0 %) (highest in Klebsiella spp.) was observed. Overall, the results of the present study strongly advocate the superiority of Elores over Piperacillin/tazobactam, Imipenem, Amoxacillin/Clavulanic acid and can be of very effective alternative to treat against the deadly multi drug resistant Gram negative bacteria.*
Keywords: *Antimicrobial resistance, β -lactamases, Gram negative bacteria, Elores*

I. Introduction

Antimicrobial resistance is a growing threat worldwide. Resistance mechanisms have been found for every class of antibiotic agents. The predominant mechanism for resistance to the beta lactam antibiotics in gram negative bacilli (GNB) is the production of extended spectrum β-lactamase (ESBL), which is responsible for the resistance to the 3rd generation of cephalosporins [1]. These enzymes catalyze the hydrolysis of the β-lactam ring of antibiotic, thereby destroying the antimicrobial activity. ESBLs have been reported worldwide in many different genera of Enterobacteriaceae and *Pseudomonas aeruginosa* [2]. ESBL producing organisms are often resistant to several other classes of antibiotics, as plasmids with gene encoding ESBLs often carry other resistance determinants. Indian subcontinent is one of the Asian country to report high rates of ESBL production. Kamath et al. [3] reported that 71.8% of blood stream infections in India were caused by Gram-negative bacteria, with *Klebsiella* species accounting for 16.4%, *Pseudomonas* species 13.6%, *E. coli* 11.8%, *Enterobacter spp.* 11.4% and *Acinetobacter spp.* 10%. Blomgran et al. [4] and Jha and Bapat [5] reported that 50% of urinary tract infections (UTI) in patients are accounted for *E.coli*. The emergence of *E. coli* and *Klebsiella species* resistance to ceftazidime and other cephalosporins seriously compromised the efficacy of these life saving antibiotics [6]. One study reported a 68% prevalence of ESBL phenotypes among *E. coli* and *K.pneumoniae* isolates, one of the highest rates reported for any country world-wide [7]. In 2007 in Asia pacific region was found to harbour plasmid borne ESBLs 62% and 75% in *E. coli* and *Klebsiella spp.* respectively [8]. ESBL production rate was 43%, 73.8%, 96% and 70% in *E. coli* and 60% in *Klebsiella spp.* in Pakistan, Iraq, Iran and India respectively in 2009, 2011 and last two were in 2010 [9-11].

Carbapenems have been the most successful β-lactam antibiotics used in the treatment of infections caused by β-lactam resistant Gram-negative bacteria. However, there have been reports of resistance to carbapenems [12,13]. Resistance to carbapenem due to the production of metallo-beta-lactamases (MBL) in Gram-negative bacteria (GNB) is an increasing international public health problem [14]. Metallo-β-lactamases (MBLs) are metalloenzymes of Ambler class B and are clavulanic acid-and tazobactam resistant enzymes. They require divalent cations of zinc as co-factors for enzymatic activity and are universally inhibited by ethylenediamine tetra-acetic acid (EDTA), as well as other chelating agents of divalent cations [15]. The metallo β-lactamase in GNB is becoming a therapeutic challenge, as these enzymes usually possess a broad hydrolysis profile that includes all β-lactam antibiotics including carbepenems [16]. IMP and VIM genes responsible for

MBL production are horizontally transferable via plasmids and can rapidly spread to other bacteria [17]. There are several mechanisms for carbapenem resistance such as the lack of drug penetration due to mutation in porins, loss of certain outer membrane proteins and efflux mechanisms [18]. In India, the prevalence of MBL range from 7.5 to 75% [19-22]. The carbapenems available for use in India are imipenem and meropenem [23]. It has also been reported that from 1989 through 2006, the proportion of *P. aeruginosa* isolates demonstrating resistance to imipenem increased from 13% to 20% [24]. Another study by Taneja et al. [25] showed 42% resistance to imipenem in urinary isolates.

Piperacillin/tazobactam is a beta-lactam/beta-lactamase inhibitor combination that has a wide range of activity against Gram-positive, Gram-negative, and anaerobic pathogens. Higgins et al. [26] reported that the addition of the β-lactamase inhibitor tazobactam to piperacillin had a small effect on raising susceptibility (30.2%), also piperacillin/tazobactam resistance rates were very high (69–75%) against *Pseudomonas*.

Amoxicillin/clavulanate is a combination of a ß-lactam with a ß-lactamase inhibitor which restores the potency of amoxicillin against strains producing ß-lactamases [27]. Combination of amoxicillin and potassium salt of clavulanic acid were effective against beta-lactamase producing gram negative strains. Current report from Moremi et al. [28] showed that 83.9% resistance was reported for amoxicillin/clavulanate among gram negative bacteria.

As the use of antibiotics such as Ceftriaxone, Piperacillin/tazobactam, Imipenem, and Amoxacillin/Clavulanic acid in the treatment of Gram-negative infections increases every day and also there is a lack of access to accurate information on regional sensitivity pattern of these antibiotics, we decided to compare the Gram-negative bacterial resistance of above antibiotics with adjuvant entity Elores (ceftriaxone/sulbactam with adjuvant EDTA).

II. Materials and Methods

2.1 Sample collection

Different clinical samples such as urine, blood, pus, sputum, bile, swab, ET secretion were collected from 563 (Five hundred and sixty three) patients suspected of bacterial infection during a period of 6 months (May to October 2014), from various diagnostic labs and hospitals of Western Uttar Pradesh and Rajasthan state of India. The collection and processing of the samples were done as per a common SOP .

2.2 Isolation and identification of microbes

All the samples were collected aseptically in sterile containers. Urine samples collected in sterile universal container were directly inoculated to the respective selective media. Other liquid specimens such as pus, sputum, bile, and endotracheal (ET) secretion collected in sufficient amount were inoculated on the different selective and non-selective culture media as per the standard microbiological techniques. Details of the culture media used for the isolation of pathogens from various clinical samples are given in Table (1). Blood samples collected in brain heart infusion (BHI) broth in a ratio of 1:5 (blood/broth) were first incubated overnight at 37°C and then subcultured on to the selective and non-selective media. All the media were incubated aerobically overnight at 37°C. Organisms were identified on the basis of colony morphology, Gram staining, motility, and biochemical reactions. Biochemical reactions were performed by inoculating the bacterial colony in a nutrient broth at 37°C for 2–3 hours.

Table 1: Selective culture medium used for isolation of different pathogens.

Pathogen	Selective media
E. coli	Eosine Methylene Blue (EMB) agar medium
A. baumannii	Leeds acinetobacter agar base medium
Klebsiella species	Hicrome Klebsiella selective agar base medium
Proteus spp.	EMB agar and Mcconkey's agar
P. aeruginosa	Citrimide agar

2.3 Antibiotic susceptibility testing

Antimicrobial susceptibility testing was done by Kirby–Bauer disk diffusion method as recommended by the Clinical Laboratory Standards Institute (CLSI) guidelines. The discs of meropenem (10 μg), Imipenem/cilastatin (10 μg), Elores disk (45 μg), Piperacillin/tazobactam (110 μg) and Amoxicillin/Clavulanate (30 μg), were procured from Himedia (Mumbai, India) and used in the study. Inoculum of 0.5 McFarland standards turbidity was prepared in a Mueller-Hinton broth (MHB, Hi-Media, Mumbai, India) from isolated colony of pathogens selected from 18–24 hour agar plates. Within 15 minutes, a sterile cotton swab was dipped into the inoculum suspension. The swab was rotated several times and pressed firmly against the inside wall of

the tube above the fluid level and inoculated on the dried surface of a Mueller-Hinton agar (MHA) plate by streaking the swab over it. For even distribution of inoculum, the swab was streaked two more times at 60° over the agar surface. After 3–5 minutes, antibiotic discs were applied and pressed down to ensure complete contact with agar surface. The discs were distributed evenly to ensure a minimum distance of 24 mm from center to center. The plates are then inverted and incubated for 16-18 hrs aerobically at 37° C within 15 minutes of disc application. Sensitivity of isolated organisms against antibiotics were reported as sensitive (S) or resistant (R) based on the breakpoints.

III. Results and Discussion

A total 563 clinical samples of urine, blood, pus, sputum, bile, swab, ET secretion processed for isolation of pathogenic bacteria. Out of the samples analyzed, 368 samples showed the presence of pathogens while in 195 samples showed no growth of organisms (Table 2). Among the samples showed the presence of pathogens, around 47.3 % samples were of urine followed by blood, pus and sputum samples which contributed to 36.0 %, 8.7% and 5.2 %, respectively. Bile, ET secretion and swab samples contributed to 1.1 %, 1.1 %, and 0.54 % respectively (Table 2).

Morphological and biochemical characterization of the samples showing bacterial growth revealed presence of 5 different Gram negative organisms such as *E. coli, Klebsiella species, P. aeruginosa, A. baumannii*, and *Proteus spp.*. The detailed profile of various organisms collected from various clinical samples is shown in Fig 1. Among the isolates, *E. coli* (61.4 %) was found to be the most dominant pathogen. Similar study performed by Sharada et al. [29] reported high rate (76.47%) of *E.coli* prevalence. In another study performed by Hamdan et al. [30] reported *E. coli* as the most common pathogen about 77.7% among Gram negative isolates. This goes with results that obtained in Tanzania where *E. coli* was 38% of the Gram-negative isolates [31]. *Klebsiella spp.* (22.0 %), and *P. aeruginosa* (14.4 %) also contributed significantly to the isolated pool of pathogens followed by *A. baumannii* (1.1 %), and *Proteus spp* (1.1 %). A similar prevalance of *Klebsiella spp.* was reported by Ananthan and Subha [32] from Chennai where they reported 23.6% of *Klebsiella spp.* from clinical isolates. In another study, Supriya et al. [33] from Nagpur have also reported the prevalence of *Klebsiella spp.* to be (25.65%). A study conducted in Aligarh tertiary care hospital has also showed the prevalence of *Klebsiella spp.* to be 30.18% from clinical samples [34]. The results of present study showed that prevalence of *P. aeruginosa* was (14.4 %) which is in accordance with the results reported by Wasnik and Tumane [35]. However Shaikh et al. [36] in his study observed 25.13% *P. aeruginosa* prevalence in clinical samples. However the isolates like *A. baumannii* (1.1 %), and *Proteus spp.* (1.1 %) contribute non-significantly in the present study, same as reported by Ejaz et al. [37] where they reported low prevalence of *A. baumannii* (1.0 %), and *P. mirabilis* (1.0 %).

Frequency of isolation of pathogenic organisms from various specimens is depicted in Table 3. *E. coli* was the most prevalent pathogen among most of the samples accounting for 42.0 % in sputum, 68.4 % urine, 60.0 % in blood, 25.0 % in bile and 25.0 % in ET secretions (Table 3). Similar results for *E.coli* was reported from Kumar et al. [38] where majority of isolates were recovered from urine (54.67%). *E. coli* was also reported to be the most prevalent organism in urine (56.57%) in a study performed by Khan et al. [39]. Kibert and Abera [40] also reported the isolation rate of *E. coli* was the highest in urine samples (45.5%). Similar results were observed by Ibrahim et al. [41] reporting high prevalence (65.1 %) of *E. coli* among the urine samples collected from urinary tract infection patients. Kumar et al. [38] reported considerable prevalence (66.67%) of *E. coli* isolated from blood. Patel et al. [42] reported high prevalence of *E coli* among sputum (45.83 %) which is in well accordance with results of the present study. *Klebsiella spp.* contributed for 42.0 % in sputum samples, 21.0 % in blood samples and 21.0 % in urine samples. Romanus and Egwu [43] reported high prevalence of *Klebsiella spp.* which was predominantly isolated from sputum (47.1%). Iroha et al. [44] also reported considerable prevalence (31.6 %) *Klebsiella spp.* in nosocomial sputum samples. Subha et al. [45] also reported considerable prevalence of *Klebsiella spp.* 42.8% in nosocomial sputum samples, (28.6%) from blood and (28.6%) from urine specimens. *P. aeruginosa* accounted for 21.0 % in sputum, 17.3 % in blood, 11.5 % in urine, 19.0 % in pus samples (Table 3). Similar results were reported by Khan et al. [46], where they reported the distribution of *P. aeruginosa* to 10.0 % in urine.

Antibiogram profile for all the pathogens isolated from various clinical samples is presented in Fig. 3 and 4. The susceptibility of *E. coli, Klebsiella spp., P. aeruginosa, A. baumannii* and *Proteus spp.* towards Elores was 81.0 %, 78.0 % , 84.0, 79 and 88.0 %, respectively, which was high when compared towards Piperacillin/tazobactam and Amoxicillin/Clavulanic acid. On the other hand *A. baumannii* showed higher resistance (75.0 %) towards Imipenem/cilastatin in comparison to other organisms i.e. *E. coli, Klebsiella spp.,* and *P. aeruginosa* (20.0 %, 33.3 % and, 28.0 %, respectively) whereas *Proteus spp.* showed 100.0 % susceptibility. A high prevalence of resistance (60%-100%) to Piperacillin/tazobactam and Amoxicillin/Clavulanic acid among all isolated Gram negative bacterial pathogens was observed. Mohammadi and Feizabadi [47] reported that the findings of their study are indicative of high resistance rates in most

microorganisms. They reported 60% Piperacillin/tazobactam resistance to most of the Gram negative organisms. Similarly amoxicillin/clavulanate resistance was about (83.9%) in a study by Moremi et al. [48]. In present study imipenem showed good activity against gram negative organisms. Similar study done by Kader and Kumar et al. [49] also showed imipenem had good activity against the ESBL-producing gram negative isolates tested (over 92% of isolates were susceptible). Previous reports [50, 51] also reported that imipenem/cilastin had the highest activity against the ESBL-producing organisms. Very recently, Sahu et al. [52] indicated higher susceptibility of Elores for *E. coli, P. aeruginosa* and *Klebsiella spp*. Earlier several authors showed that the overall resistance to various generations of cephalosporins was high on account of the production of ESBLs by the bacteria [53,54]. Hence, addition of sulbactam with adjuvant EDTA to ceftriaxone monotherapy significantly reduced the percentage resistance and increased the percentage susceptibility against all the organisms (Fig. 4). According to a previous study conducted in India for the treatment of skin and skin structure infection (SSSIs) and bone and joints infections (BJIs) more than 80 % of the studied patient were clinically cured with ceftriaxone+sulbactam with adjuvant EDTA (Elores) [22].

IV. Conclusion

The bacterial susceptibility and resistance profile of all isolates in this study have shown that Elores and Imipenem remain the most effective drugs against gram negative pathogens, suggesting that use of Elores over other antibiotics should be preferred. However there is a need to emphasize on the rational use of antimicrobials and strictly adhere to the concept of reserve drugs to minimize the misuse of available antimicrobials. In addition, regular antimicrobial susceptibility surveillance is essential.

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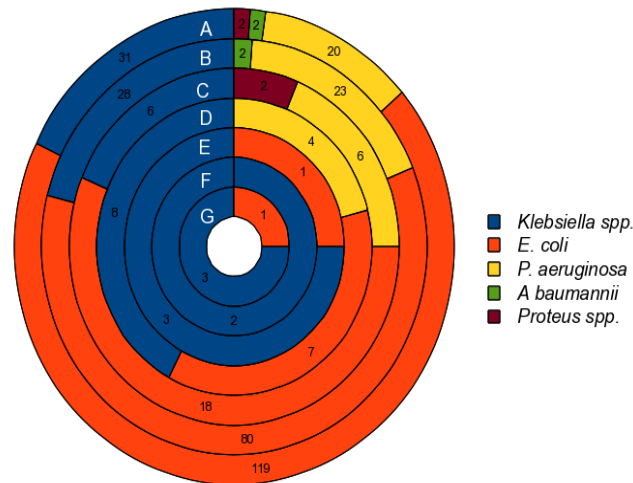
A-Urine; **B**-Blood; **C**-Pus; **D**-Sputum; **E**-Bile; **F**-Swab; **G-ET secretions**

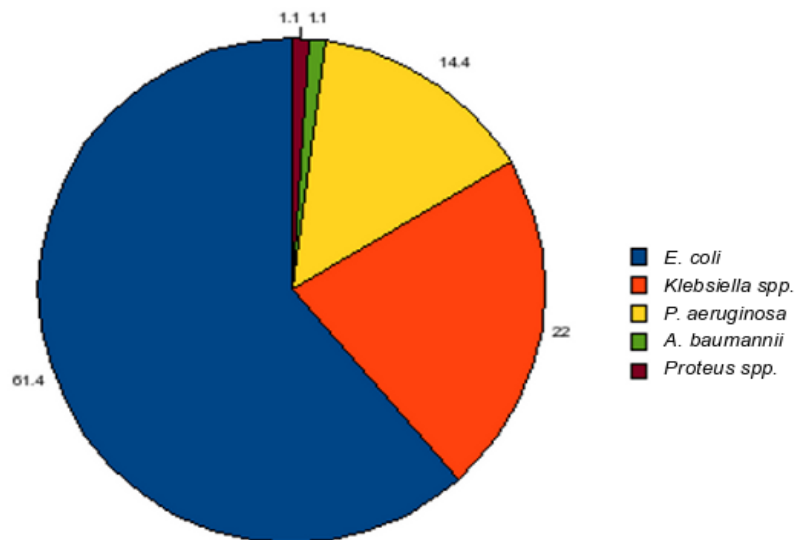**Figure 1:** Profile of different clinical isolates isolated from various samples

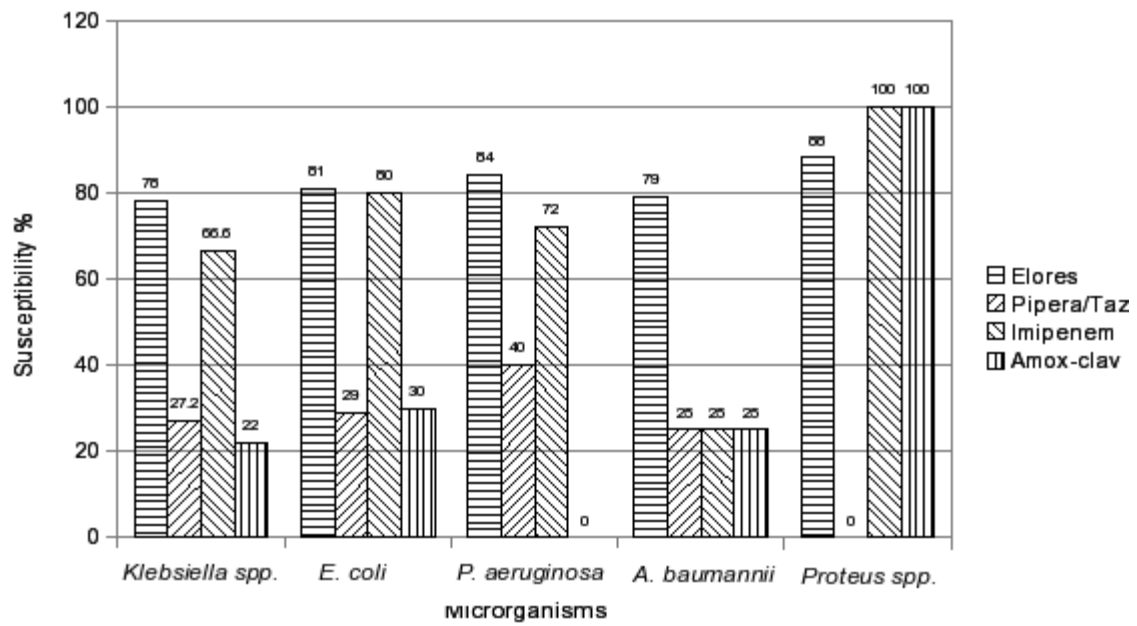**Figure 2:** Prevalence of various pathogen

Figure 3: Susceptibility pattern of Gram negative pathogens isolated
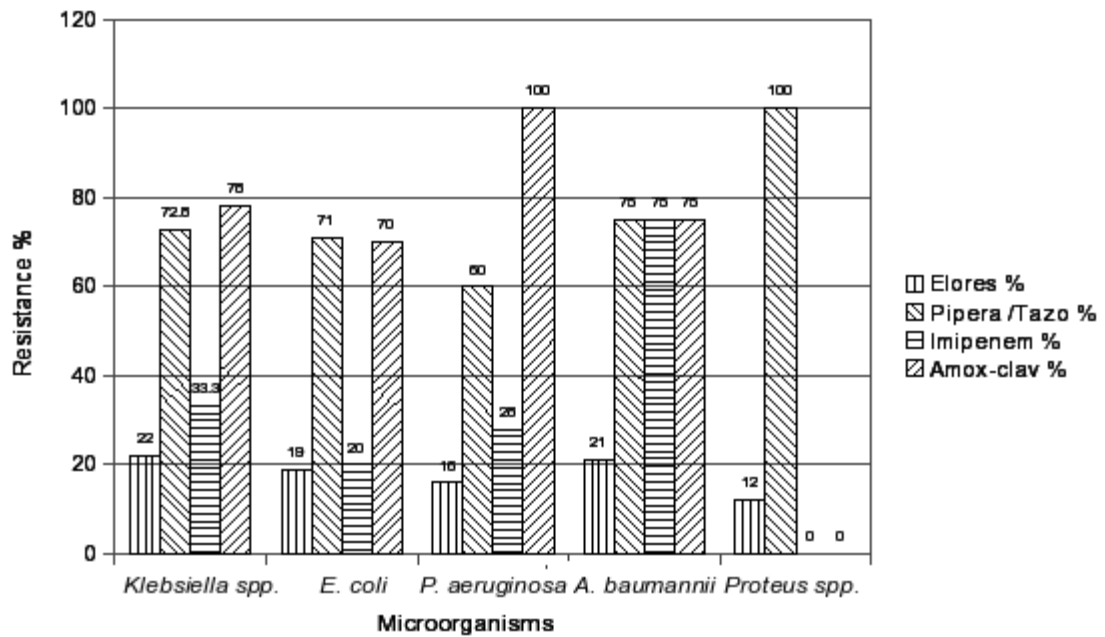
Figure 4: Resistance pattern of Gram negative pathogens isolated

Table 2: A profile of clinical samples used as a source of the pathogenic isolates.

Sr. No.	Clinical samples	Total	Number of samples showing growth of pathogens (%)	Number of samples not showing growth of pathogens
1	Urine	250	174 (47.3)	76
2	Blood	196	133 (36)	63
3	Pus	54	32 (8.7)	22
4	Sputum	29	19 (5.2)	10
5	Bile	10	4 (1.1)	6
6	Swab	11	2 (0.54)	9
7	ET secretion	13	4 (1.1)	9
	Total	**563**	**368**	**195**

Table 3. Prevelence of different clinical isolates in different samples.

Samples	No. of Isolates	Clinical isolates				
		Klebsiella spp. (n; %)	*E. coli* (n;%)	*P. aeruginosa* (n;%)	*A. baumannii* (n;%)	*Proteus spp.* (n;%)
Urine	**174**	31 (17.8)	119 (68.4)	20 (11.5)	2 (1.1)	2 (1.1)
Blood	**133**	28 (21)	80 (60)	23 (17.3)	2 (1.5)	0
Pus	**32**	6 (19)	18 (56.3)	6 (19)	0	2 (6.3)
Sputum	**19**	8 (42)	7 (42)	4 (21)	0	0
Bile	**4**	3 (75)	1 (25)	0	0	0
Swab	**2**	2 (100)	0	0	0	0
ET secretion	**4**	3 (75)	1 (25)	0	0	0