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Characterization of *Klebsiella terrigena* strains from humans: haemagglutinins, serum resistance, siderophore synthesis, and serotypes

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SUMMARY

Klebsiella terrigena is very rarely isolated from humans; as yet, its clinical significance is uncertain. The aim of the present study was to evaluate whether this species is able to express putative virulence factors. A total of 72 faecal ($n = 50$) and clinical ($n = 22$) *K. terrigena* isolates was investigated and compared with faecal and clinical strains of *K. pneumoniae*. Mannose-sensitive haemagglutination (MSHA) was observed less often in *K. terrigena* (64–74%) than in *K. pneumoniae* strains. In contrast, the incidence of mannose-resistant haemagglutinin indicative of type 3 pili (MR/K-HA) (77–94%), serum resistance properties (10–23%), and production of enterobactin (100%) was similar in both species. None of the *K. terrigena* isolates were able to synthesize aerobactin; however, the frequency of aerobactin synthesis in *K. pneumoniae* was also only 5%. Serotyping showed capsular types K5 and K70 to be predominant. The virulence-associated serotype K2 was common in both *K. terrigena* and *K. pneumoniae* isolates. Taken together, the present results suggest that *K. terrigena* and *K. pneumoniae* are indistinguishable with respect to the expression of virulence factors.

INTRODUCTION

Klebsiella spp. are nosocomial pathogens commonly encountered in urinary tract infections (UTI), septicaemia, or respiratory tract infections. Primarily, these bacteria attack immuno-compromized and hospitalized patients suffering from severe underlying diseases. *Klebsiella* species are among the eight most frequently reported pathogens in hospitals, causing 3–7% of all nosocomial infections [1]. Klebsiella infections are caused mainly by *K. pneumoniae*, the medically most important member of this genus. To a much lesser extent, *K. oxytoca* has been isolated from human infections.

In 1981 a new species, *K. terrigena*, formerly designated 'group L' was proposed by Izard and colleagues [2]. *K. terrigena* is considered to be an environmental *Klebsiella* species as it has been isolated mainly from soil and water [3]. There have been only rare reports on the isolation of these bacteria from human specimens, and the clinical significance of this species is as yet unknown. A study on *K. terrigena* colonization of the human bowel comprising 5377 stool specimens from healthy persons detected a faecal carriage rate of 0·9% [4]. Another investigation on the incidence of *K. terrigena* among clinical klebsiella isolates demonstrated *K. terrigena* in 0·4% of 2355 indole-negative klebsiella isolates [5]. Most of these isolates were recovered from the respiratory tract. As yet, *K. terrigena* has not been demonstrated as a causative agent of human infectious diseases, and the pathogenicity of this species remains unclear.

As a non-pathogenic *Klebsiella* species, *K. terrigena* should express fewer virulence factors than the more pathogenic *K. pneumoniae*. Chief among the factors thought to contribute to the pathogenicity of klebsiella [6] is the production of capsular polysaccharides.

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Seventy-seven different capsular types have been reported [7], some serotypes appearing to be more virulent than others [8, 9].

Apart from the production of capsules, a number of other virulence factors have been described. Most strains of *K. pneumoniae* produce two different types of fimbriae or pili that mediate adhesion to host cells. Type 1 (or common) pili cause mannose-sensitive haemagglutination (MSHA), and play an important role in UTI [10–12]. Type 3 fimbriae mediate mannose-resistant and klebsiella-like agglutination of tanned erythrocytes (MR/K-HA), and are reported to correlate with catheter-associated bacteriuria caused by *Providencia stuartii* [13].

Another characteristic regarded as a virulence factor is their ability to resist the bactericidal effect of human serum. In *K. pneumoniae*, serum resistance properties are more common among isolates from clinical specimens than among faecal or environmental strains [14].

Klebsiella isolates have also been shown to produce high-affinity iron-chelating compounds, called siderophores [15–17]. While the role of the catechol-type siderophore enterobactin in virulence is still uncertain, the contribution of the hydroxamate-type aerobactin has been clearly demonstrated [18].

The present study was undertaken to evaluate whether *K. terrigena* expresses any of the aforementioned known virulence factors. Our working hypothesis was that the greater the pathogenicity of a given *Klebsiella* species, the higher the percentage of strains expressing putative virulence factors. If *K. terrigena* is a rather non-pathogenic species, the incidence of such features should be rare or entirely absent. For this purpose, faecal and clinical isolates of *K. terrigena* were compared with a group of clinical and faecal *K. pneumoniae* isolates.

METHODS

Bacterial strains

A total of 72 *K. terrigena* human isolates was investigated. These strains were obtained from the faeces of healthy persons ($n = 50$) or from clinical specimens ($n = 22$). Fifteen (68%) of the clinical isolates were obtained from respiratory secretions, 1 strain from blood culture, 2 strains from urine, and 4 isolates from wounds. Most of the strains ($n = 60$) have been described previously [4, 5]. All isolates were identified by the API20E system (API BioMérieux, Germany) plus additional tests (fermentation of melezitose and L-sorbose, gas production from lactose at 44·5 °C, growth at 10 °C, pectate degradation, utilization of m-hydroxybenzoate and hydroxy-L-proline) as described previously [4].

The comparison group of 316 *K. pneumoniae* isolates consisted of 109 human faecal strains that have been described elsewhere [12, 19] and of 207 human clinical isolates. The clinical isolates were obtained from human UTI, bacteremia, pneumonia, and wound infections. All strains were stored until use in brain heart infusion broth/30% glycerol at −80 °C. *E. coli* strains H1939, H1887, H1886, and K311 were kindly provided by K. Hantke (University of Tübingen, Germany). *E. coli* F205 was a gift of P. Williams (University of Leicester, England).

Capsule typing

The isolates were serotyped by the capsular swelling method as described by Ullmann [20]. Polyvalent anticapsular sera were used for screening and monospecific sera for typing.

Haemagglutination assay

The expression of type 1 fimbriae (MSHA) and type 3 pili (MR/K-HA) was examined as described previously [21]. MSHA was assessed on guinea-pig erythrocytes. MR/K-HA was determined on tanned ox red blood cells. Bacteria were grown statically at 48 h intervals. Fifty μl of bacterial suspensions (approx. 10^{11} bacteria/ml) and 50 μl of erythrocytes (5×10^8/ml) were mixed in rocked porcelain tiles, and observed for 3 min at room temperature. Agglutination was finally read after further incubation at 4 °C.

Determination of siderophore production

For detection of enterobactin and aerobactin production the cross-feeding bioassay of Hantke [22] was performed as described elsewhere [16]. Nutrient agar supplemented with 2,2′-dipyridyl (200 μM) served as iron-restricted agar medium. *E. coli* H1887 (ColV$^-$, Aer$^-$, Iut$^+$, FepA$^-$, Fiu$^-$, Cir$^-$, aroB) was used as indicator strain for aerobactin production, and strain H1939 (FepA$^+$, Fiu$^-$, Cir$^-$, FhuA$^-$, FhuB$^-$, aroB) for enterobactin. Aerobactin production was counterchecked by means of *E. coli* strain H1886 which is the Iut$^-$ parent strain of H1887. Strain K311 (pColV-K311) served as positive control in the aerobactin test. Each isolate was tested twice.

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Serum bactericidal assay

The susceptibility of bacteria to human serum was determined by the method of Hughes and colleagues [23] as slightly modified [14]. Bacteria were diluted to 2×10^6 cells/ml in physiological saline. Twenty-five μl of bacterial suspensions and 75 μl of normal human serum (NHS) were put into microtitre trays, mixed, and incubated at 37 °C. Viability was determined immediately and after 1, 2 and 3 h of incubation: After mixing, samples were taken and serial dilutions were plated on brain heart infusion agar for colony counts. Responses were graded from 1 to 6 according to Hughes and colleagues [23]: grade 1, viable counts (VC) after 1 and 2 h were $< 10\%$ of the inoculum, after 3 h $< 0{\cdot}1\%$; grade 2, VC after 1 h were 10–100%, after 3 h $< 10\%$; grade 3, VC after 1 h were $> 100\%$, after 2 and 3 h $< 100\%$; grade 4, VC after 1 and 2 h were $> 100\%$, after 3 h $< 100\%$; grade 5, VC after 1, 2 and 3 h were $> 100\%$, but VC fell at some time during the 3 h period; grade 6, VC after 1, 2 and 3 h were $> 100\%$ of the inoculum and rose throughout the 3 h period. An isolate was classified as being highly sensitive (grades 1 and 2), intermediately susceptible (grades 3 and 4), or as serum resistant (grades 5 and 6). Each strain was tested three times.

Statistical analysis

The significance of differences between groups of bacteria was evaluated by Yates corrected χ^2 for 2×2 contingency tables. Comparison of medians was performed by the non-parametric ANOVA test of Kruskal–Wallis, and by the Mann–Whitney test.

RESULTS

A total of 72 human faecal and clinical *K. terrigena* isolates was investigated with respect to the expression of factors thought to contribute to the virulence of klebsiella. The incidence of strains expressing such markers was compared with that observed among 316 faecal and clinical *K. pneumoniae* isolates.

MSHA

Mannose-sensitive haemagglutination indicative of type 1 fimbriae could be detected in most *K. terrigena* isolates (Table 1). The incidences of MSHA expression in faecal (74%) and clinical (64%) strains were not significantly different. MSHA in both *K. terrigena* groups, however, was significantly less frequent than in the corresponding faecal (89%) or clinical (86%) *K. pneumoniae* isolates ($P < 0{\cdot}025$).

MR/K-HA

Mannose-resistant, klebsiella-like haemagglutination indicative of type 3 pili was significantly more frequent among faecal strains of *K. terrigena* (94%) and *K. pneumoniae* (89%) than among clinical isolates (77 and 70%, respectively) of both species ($P \leqslant 0{\cdot}05$). The incidence of MR/K-HA in both *K. terrigena* groups was similar to that observed in the corresponding *K. pneumoniae* groups (Table 1).

Serum resistance properties

The isolates were examined over a period of 3 h with respect to their susceptibility to the bactericidal activity of human serum. Serum resistance (grades 5 and 6) was observed among 10–25% of the *K. pneumoniae* and *K. terrigena* isolates (Table 1). Faecal strains of both species were less often serum resistant than were clinical isolates, however, differences between the isolation source and between *K. pneumoniae* and *K. terrigena* were not statistically significant ($P > 0.05$).

Siderophore production

All *K. terrigena* isolates were able to synthesize the catechol-type siderophore enterobactin (Table 1). Similarly, except for two clinical isolates, all *K. pneumoniae* strains produced enterobactin under iron-limited conditions. In contrast, very few of the strains investigated synthesized the hydroxamate-type siderophore aerobactin. But while 5% each of the faecal and clinical *K. pneumoniae* isolates were aerobactin-positive, aerobactin production was completely absent in *K. terrigena* (Table 1). Interestingly, the majority of *K. terrigena* strains synthesized a hydroxamate-type siderophore (Table 1) that was not aerobactin as proven by the indicator strain *E. coli* H1886.

Capsule types

As performed by the capsule swelling reaction, K typability of *K. terrigena* strains was excellent, being similar to that of *K. pneumoniae* strains. Altogether, 65 different capsule types could be detected. With respect to *K. terrigena*, capsule typing revealed 38 different serotypes, fewer than 6% of the strains being untypable. The distribution of the capsular types most often found is shown in Table 2. The most frequent

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Table 1. *Distribution of fimbriae, serum resistance properties, and siderophores among faecal and clinical isolates of* K. terrigena *and* K. pneumoniae

	No. of isolates (%) positive			
	K. terrigena		*K. pneumoniae*	
Feature	Faecal ($n = 50$)	Clinical ($n = 22$)	Faecal† ($n = 109$)	Clinical ($n = 207$)
MSHA (type 1 fimbriae)	37 (74)	14 (64)	97 (89)	177 (86)
MR/K-HA (type 3 fimbriae)	47 (94)	17 (77)	97 (89)	145 (70)
Serum resistance*	5 (10)	5 (23)	18 (17)	52 (25)
Production of				
Enterobactin	50 (100)	22 (100)	109 (100)	205 (99)
Hydroxamates	36 (72)	14 (64)	11 (10)	26 (13)
Aerobactin	0 (0)	0 (0)	5 (4·6)	11(5·3)

* Grades 5 or 6.
† Data from references [14, 16, 21].

Table 2. *Distribution of capsular types among faecal and clinical isolates of* K. terrigena *and* K. pneumoniae

	No. of isolates (%)			
	K. terrigena		*K. pneumoniae*	
Capsule type	Faecal ($n = 50$)	Clinical ($n = 22$)	Faecal* ($n = 109$)	Clinical ($n = 207$)
K5	2 (4·0)	3 (13·6)	1 (0·9)	4 (1·9)
K70	5 (10)	— (0)	1 (0·9)	— (0)
K2	1 (2·0)	2 (9·1)	6 (5·5)	28 (13·5)
K18	1 (2·0)	2 (9·1)	1 (0·9)	8 (3·9)
K32, K44	— (0)	4 (9·1)	2 (0·9)	2 ($\leqslant$ 0·5)
K33	— (0)	2 (9·1)	9 (8·3)	3 (1·4)
K42	3 (6·0)	2 (9·1)	— (0)	1 (0·5)
K14	4 (8·0)	— (0)	4 (3·7)	9 (4·3)
K61	3 (6·0)	— (0)	2 (1·8)	2 (1·0)
K12, K38	2 (2·0)	— (0)	15 ($\leqslant$ 8·3)	5 ($\leqslant$ 2·4)
K7, K37	— (0)	— (0)	13 ($\leqslant$ 6·4)	5 ($\leqslant$ 1·4)
Other K types	each < 5%		each < 5%	
Untypeable	3 (6·0)	1 (4·5)	4 (3·7)	16 (7·7)

* Data from ref. [19].

serotype in *K. terrigena* was K5 among clinical isolates (13·6%) and K70 among faecal strains (10%). In contrast, both K antigens were observed rarely in *K. pneumoniae*. However, serotype K2 strains that were commonest among clinical *K. pneumoniae* isolates (13·5%) were also common among clinical *K. terrigena* isolates (9%).

Cumulative expression of virulence factors

To determine whether *K. terrigena* isolates express fewer virulence factors than *K. pneumoniae* strains, the factors expressed by each strain (MSHA, MR/K-HA, serum resistance, enterobactin production, and aerobactin synthesis) were added up to get the cumulative number of virulence factors expressed per strain. A box-and-whisker plot of these data is shown in Figure 1. The mean number of virulence factors expressed by isolates of each species (2·6 by *K. terrigena vs.* 2·9 by *K. pneumoniae*) did not differ significantly. Likewise, faecal *K. terrigena* isolates did not express significantly fewer virulence factors than did clinical strains.

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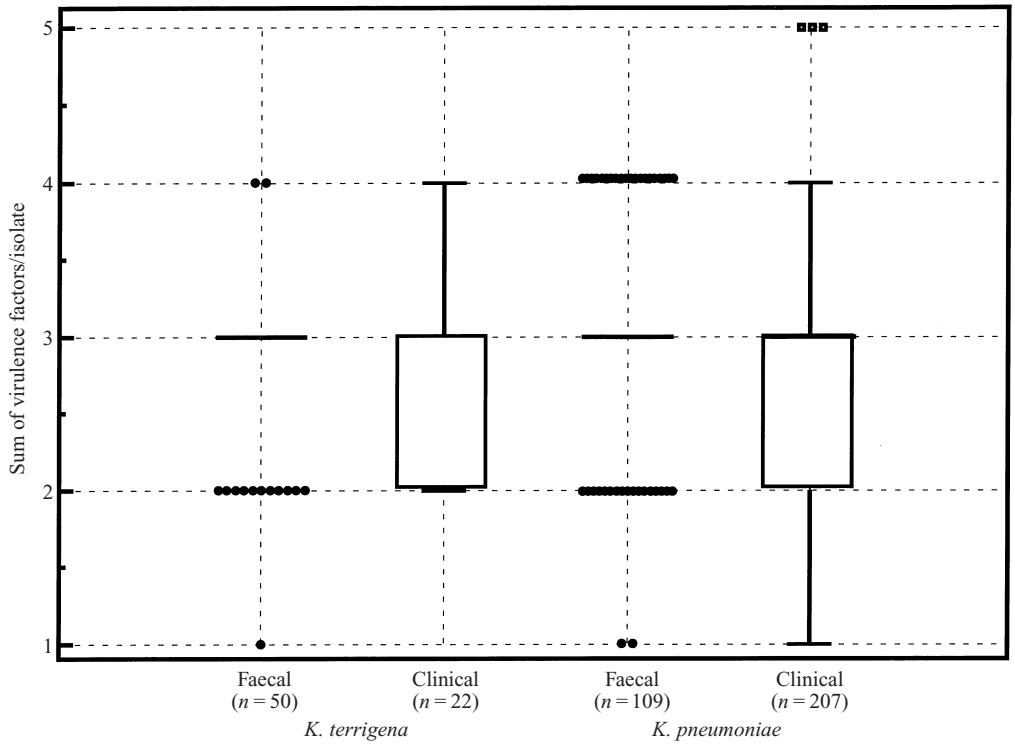

Fig. 1. Blot-and-whisker plot of the cumulative number of virulence factors detected in clinical and faecal isolates of *K. terrigena* and *K. pneumoniae*. Boundaries of the boxes represent the 25th and 75th percentiles (interquartile range). Whiskers represent the ranges of values that lie within 1·5 and 3 times the interquartile range. Square markers indicate values between 1·5 and 3 times, closed circles outlying values > 3 times the interquartile range.

DISCUSSION

Even nearly 20 years after its first description, reports on the isolation of *K. terrigena* from human clinical specimens are remarkably rare. The obvious lack of association of this species with human hosts might be due to the inability of conventional laboratory differentiation procedures to identify *K. terrigena*. However, an epidemiological study on the frequency of this species in clinical specimens reported only 0·4 % of all klebsiella isolates as being *K. terrigena* [5]. Thus, this species appears to have no or only a very low clinical significance. Bearing this in mind, we developed our working hypothesis that *K. terrigena*, being a non-pathogenic *Klebsiella* species, should express putative virulence factors to a much lesser degree, or not at all, than do *K. pneumoniae*.

As it turned out, with respect to the production of type 1 pili, faecal and clinical *K. terrigena* isolates were indeed significantly less often MSHA-positive than the corresponding faecal and clinical *K. pneumoniae* strains. Type 1 pili are regarded as the main adhesin of klebsiella. Their contribution to bacterial virulence is thought to arise from their enabling the bacteria to bind to the urogenital, intestinal, and respiratory tracts of the host [24–26]. In klebsiella, the significance of type 1 pili in the pathogenesis of infection has been demonstrated in different animal models [10, 11, 27]. Thus, the observed lower frequency of type 1 pili-positive *K. terrigena* isolates compared to *K. pneumoniae* suggests *K. terrigena* is less able to bind to and colonize host mucosal surfaces. As adherence is a critical first step in the infectious process, low ability to attach to host cells reflects low bacterial virulence. However, even if *K. terrigena* strains express type 1 pili significantly less often than do *K. pneumoniae* isolates, it should be remembered that the majority of faecal and clinical *K. terrigena* isolates (64–74 %) were in fact able to produce type 1 pili.

In contrast to the results on type 1 pili, no significant differences were detected in the incidences of type 3 pili production between *K. terrigena* and *K. pneumoniae*. Type 3 pili have been found to bind to human epithelial cells of the respiratory tract, to endothelial cells, and to uroepithelial cells [28–30]. However, the role of type 3 pili adherence in the pathogenesis of infection is largely unknown. Data from a study on

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klebsiella UTI suggest that these fimbriae are not associated with human UTI [12]. So far, the only evidence for a correlation between type 3 pili and disease was the finding that expression of these pili was associated with the persistence of *Providencia stuartii* in catheter-associated bacteriuria [13].

In addition to bacterial adherence properties, resistance to the bactericidal effect of serum is thought to contribute to the virulence of klebsiella [6]. Killing of susceptible bacteria by serum is mediated by complement and follows activation of either the classical or the alternative pathway. Capsular antigens, lipopolysaccharides, and outer membrane proteins have been shown to mediate serum resistance in klebsiella; however, to date the exact nature of resistance to serum killing is not completely understood [31–34]. Clinical isolates of enterobacteria are often serum-resistant while most commensal Gram-negative bacteria are usually sensitive to the bactericidal effect of human serum [35, 36]. In *K. pneumoniae*, clinical isolates were found to be serum-resistant significantly more often than faecal strains [14]. The present findings do not confirm this observation because the differences in the frequencies of serum-resistance observed between clinical and faecal isolates of *K. pneumoniae* and *K. terrigena* did not prove as statistically significant. Furthermore, in both species comparable incidences of serum-resistant strains were found, suggesting that the ability of *K. terrigena* to express serum resistance properties is equal to that of *K. pneumoniae*.

The human host represents an iron-restricted environment for bacteria. Pathogenic bacteria may overcome this restriction by the excretion of low-molecular-weight iron-binding chelators, called siderophores. Two types of siderophores have been described in klebsiella: the catechol-type chelator enterobactin and the hydroxamate-type siderophore aerobactin. Enterobactin is regarded as the main iron-uptake system of enterobacteria, being produced by most of the strains [37]. The ability to synthesize enterobactin is also a uniform characteristic within the genus *Klebsiella*, and is so regardless of the source of isolation [16, 38]. In accordance with these findings, enterobactin production was observed in almost all klebsiella strains investigated in the present study, showing that *K. terrigena* and *K. pneumoniae* are identical with respect to this trait. While studies on the role of enterobactin in bacterial virulence led to conflicting results [39, 40], the association of aerobactin production and klebsiella virulence has been clearly demonstrated [18]. However, aerobactin synthesis is not very common in klebsiella strains and was observed at similarly low frequencies among clinical, faecal, and environmental isolates [16, 17]. In the present study, none of the *K. terrigena* isolates investigated was found to be aerobactin-positive, suggesting a species-specific inability to synthesize aerobactin. However, most *K. terrigena* strains produced a hydroxamate-type siderophore other than aerobactin. In contrast, only a small percentage of the examined *K. pneumoniae* isolates synthesized this type of siderophore. Presently, the nature of this siderophore and its possible significance to the bacteria are unknown. This siderophore has not been reported in the genus *Klebsiella* so far and seems to be a specific character of *K. terrigena*.

Capsular antigens are considered to be a major factor in the pathogenicity of klebsiella [6]. Among the 77 serological types, particular K antigens are associated with the site of infection, and great differences in virulence have been observed among different capsular types [8, 9, 41]. Strains expressing capsular type K2 or K5 have reported to be more virulent than those with other serotypes [9]. Serotype K2 is frequently observed in clinical *K. pneumoniae* isolates while K5 is not common [19, 42]. In the present study, both serotypes were commonly found among clinical, but not among faecal isolates of *K. terrigena*. Thus, the rather high incidence of *K. terrigena* strains expressing capsular types thought to confer klebsiella virulence properties does not support our working hypothesis of a low pathogenicity of *K. terrigena*.

However, bacterial virulence very probably is not restricted to a single feature but is multifactorial in nature. We therefore examined whether *K. terrigena* and *K. pneumoniae* strains differed in the average number of virulence factors they expressed. The expression of the factors 'MSHA', 'MR/K-HA', 'serum resistance', 'enterobactin production', or 'aerobactin synthesis' was added up to get the cumulative number of virulence factors produced by each strain. The result showed, however, that *K. terrigena* did not express fewer of the virulence factors examined than did *K. pneumoniae* strains.

In conclusion, our working hypothesis that *K. terrigena* is a non-pathogenic *Klebsiella* species was not confirmed by our findings on the frequency of virulence factors expressed by the isolates. *K. terrigena* appears to be just as able as *K. pneumoniae* to express virulence factors. This observation is in striking

contrast to our recent epidemiological findings indicating a minimal role of *K. terrigena* in human diseases. The question arises whether the factors chosen in this study are appropriate for determining klebsiella virulence. However, recent findings on an association between the incidence of these features and the source of klebsiella isolates [12, 14, 21] in our opinion justify the choice made. Moreover, the significance of these factors has been demonstrated in other enterobacterial genera as well. However, presently it cannot be ruled out whether other, as yet unknown factors, might be significant for klebsiella virulence.

Another point to be taken into account is the source of the clinical *K. terrigena* isolates. The great majority of isolates (68%) were obtained from respiratory secretions. Only five strains were isolated from normally sterile body sites (blood, wounds). Moreover, four strains only were from monomicrobial specimens. Thus, the clear distinction between colonization and disease could be achieved only rarely.

Nevertheless, the question why *K. terrigena* is found so rarely in human clinical specimens remains unsolved. This species is still thought to occur primarily in the environment. However, the frequency and distribution of *K. terrigena* in the environment is unknown. Data on the incidence and distribution of this *Klebsiella* species in natural sources such as soil, surface waters, or botanical habitats are needed.

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